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SELECTIVE PLATING MEDIUM FOR *LEUCONOSTOC* IN MIXED LACTIC CULTURES

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SUMMARY

Thirty-six strains of starter organisms (*Leuconostoc* and *Streptococcus*) were tested for their susceptibility to various antibiotics and sulfa drugs. Tetracycline hydrochloride was found to have less inhibitory effect on leuconostocs than on streptococci. Consequently, a simple selective plating medium containing tetracycline (0.15 μg/ml) in a tomato juice agar base was developed. Five experimentally mixed starters of known composition and seven commercial multiple-strain lactic starters were used to evaluate the effectiveness of the medium. The mixed starters were plated in control and in the experimental agar and incubated at 30 C for up to 72 hr. Representative colonies were isolated from the tetracycline agar after 48 hr and after 72 hr and were identified by growth in litmus milk and by biochemical tests. Results showed an incubation period of 48 hr to be optimum for best selectivity of leuconostocs, because a few strains of *Streptococcus lactis* were not inhibited on further incubation.

The actual role of leuconostocs in starter cultures has not been fully evaluated, because of an inability to determine accurately their presence and their ratios to the streptococci in any given mixed culture or product. Common separation methods such as incubation until death of the less acid-tolerant streptococci do not reveal ratios and are time-consuming. None of the numerous attempts to develop selective or differential media (1, 2, 5, 6) can be classified as successful. The recent medium of Galesloot et al. (4) was tested with a limited number of mixed cultures with some degree of success. However, the method involves considerable preparation and requires up to a six-day incubation period. Most proposed methods have utilized an indicator to differentiate between the high acid-producing streptococci and the low acid-producing leuconostocs. These methods have proven to be completely unreliable in cultures containing small numbers of leuconostocs for two reasons: (1) The leuconostocs would be eliminated by the dilution necessary to obtain a countable plate, and (2) plates containing large numbers of streptococci are usually completely acidified by diffusion of the acid throughout the agar. Since most commercial cultures contain less than 10% leuconostocs, it is obvious that a successful selective medium must be based on inhibition of the streptococci accompanied by normal development of leuconostocs. Preliminary studies in this laboratory had indicated that leuconostoc strains were more resistant to certain antibiotics than the lactic streptococci. This paper presents the results of a study in which antibiotic inhibition is utilized to develop a selective plating medium for *Leuconostoc.*

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MATERIALS AND METHODS

Cultures. In all, 19 strains of *Streptococcus,* 17 strains of *Leuconostoc,* and seven commercial, multiple-strain cultures were employed in this study. Most of the single strains were from our stock culture library. Others were either isolated from commercial starters or obtained from other laboratories. The streptococci were maintained in litmus milk and the leuconostocs in litmus milk fortified with 1% each of glucose and yeast extract. The commercial starters were obtained from various starter laboratories in lyophilized form and were reconstituted and incubated according to the supplier's instructions.

Preparation of antibiotic solutions. Stock solutions of tetracycline were prepared as recommended by the Division of Antibiotics, Food and Drug Administration (3), by dissolving 100 mg of pure powdered tetracycline hydrochloride in 100 ml of 0.1 N hydrochloric acid. Thus, the solutions contained 1,000 μg/ml. Stock solutions should be stored in a refrigerator for no longer than 2 wk. Aliquots may be stored in frozen state for one to two months, but should not be refrozen.

Preparation of media. The basal medium, tomato juice agar, was prepared as follows:

Clarified tomato juice (200 ml) was adjusted to pH 6.8-6.9 and mixed with 5 g each of tryptone, tryptose, and yeast extract, and 15 g of agar. The mixture was diluted with 800 ml of distilled water and steamed in an Arnold steamer until dissolved. It was then dispensed in 250-ml portions and autoclaved at 15-lb pressure for 15 min. Tomato juice broth was prepared as above, without agar, and was dispensed in 100-ml bottles. Trials with other basal media revealed that many were unacceptable for a variety of reasons; hence, the use of others is not recommended without preliminary investigation.

Turbidity measurements. The optical densities of cultures in tomato juice broth were measured with a Bausch and Lomb Spectronic 20 Spectrophotometer,[1] using a 600-mμ wavelength. Control tubes of sterile medium were used to adjust the machine to 100% transmittance.

EXPERIMENTAL PROCEDURE AND RESULTS

(a) *Effectiveness of tetracycline broth.* Appropriate amounts of tetracycline stock solution were added to sterile tomato broth to yield concentrations of 0.05, 0.1, 0.15, and 0.2 μg/ml. The media were then dispensed aseptically into sterile 16- by 150-mm culture tubes, 10 ml per tube. Individual tubes of each concentration were inoculated with 0.1 ml of each of the experimental strains. Turbidity was measured after incubation at 30 C for 24 hr. A tube was inoculated with 1% of culture and used immediately as a control to adjust the spectrophotometer. The percentages of transmittance are given in Tables 1 and 2. Results show that a tetracycline level of 0.15 μg/ml markedly inhibited the streptococci and had practically no effect on the leuconostocs.

(b) *Effectiveness of tetracycline agar.* Melted tomato juice agar was cooled to 44-45 C and sufficient tetracycline stock solution added aseptically to yield 0.15 μg/ml. Each test strain was plated by the pour method in tetracycline agar and in basal control agar and incubated at 30 C for 48 hr. Representative data are shown in Table 3. All of the streptococci were inhibited, except *Streptococcus lactis* Strain 924, which developed a low count of pinpoint colonies. The leuconostocs were not affected by the tetracycline, except Strains 122-5 and H-2-5-10, which were unusually slow-growing and required a slightly longer incubation period.

(c) *Effectiveness of tetracycline on laboratory-combined cultures.* To determine the selectivity of the antibiotic on cultures containing both streptococci and leuconostocs, five combinations containing different strains of

[1] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

TABLE 1

Turbidity of 24-hr cultures of streptococci in tetracycline broth [a]

Strain	Tetracycline levels				
	0	0.05	0.1	0.15	0.2
	(μg/ml)				
Control	100.0	100.0	100.0	100.0	100.0
ML-1	28.2	94.1	100.0	100.0	100.0
ML-3	22.0	26.5	42.3	95.2	99.4
C 10	24.6	28.0	46.2	97.8	100.0
C 13	30.8	93.7	100.0	100.0	100.0
C 13 L	24.4	32.5	96.0	94.3	99.5
K	41.0	73.7	95.5	100.0	100.0
KH	27.6	34.4	95.2	100.0	100.0
C 1	<1.0	4.0	40.1	97.2	100.0
C 3	24.1	27.2	97.5	100.0	100.0
C 6	27.6	45.0	99.1	100.0	100.0
E8	24.8	40.6	94.0	100.0	100.0
924	26.0	30.0	59.0	98.0	100.0
926	24.5	26.1	76.0	97.1	100.0
Z 8 L	23.1	25.5	83.2	95.0	99.6
R 1	3.0	13.1	64.5	96.5	98.2
US-3	<1.0	4.0	22.2	90.1	95.0
H-1	29.0	51.1	96.2	100.0	100.0
H-4	30.0	99.4	100.0	100.0	100.0
HP	30.1	45.2	98.1	100.0	100.0

[a] Expressed as per cent transmittance.

TABLE 2

Turbidity of 24-hr cultures of *Leuconostoc* in tetracycline broth [a]

Strain	Tetracycline levels				
	0	0.05	0.1	0.15	0.2
	($\mu g/ml$)				
Control	100.0	100.0	100.0	100.0	100.0
AND	1.0	1.0	1.0	1.0	2.5
ZYMR	1.0	1.0	3.9	3.4	5.0
LYSLE	1.0	1.0	1.0	1.0	3.1
CR-6	1.0	1.0	1.0	1.1	2.2
Cr-101	1.0	1.0	1.0	5.1	21.0
8356	1.0	1.0	7.0	9.1	13.0
8357	1.0	1.0	1.0	5.4	7.1
8358	25.0	25.0	26.2	26.1	33.4
8359	1.0	1.0	2.4	4.1	10.1
I-11	1.0	1.0	1.0	1.0	5.1
H-3	1.0	1.0	1.0	1.0	1.0
L-169	1.0	1.0	1.0	3.4	10.3
9135	1.0	1.0	1.0	1.0	2.1
E-11	1.2	2.0	8.1	6.1	6.5
L-2-25	1.1	1.0	1.0	1.0	4.3
H2-5-10	50.0	51.1	64.4	79.1	89.9
122-5	39.5	39.5	41.4	43.2	57.4

[a] Expressed as per cent transmittance.

each organism were prepared. Each combination contained 95% of streptococci and 5% of leuconostoc and was prepared by mixing 2 ml of a single strain of streptococci and 0.1 ml of a single strain of leuconostoc in 8 ml of sterile water. Appropriate dilutions of each combination were plated immediately in tetracycline agar and in control agar and incubated at 30 C for 48 hr. The pure strains composing the combinations were plated individually to serve as controls.

Table 4 shows that the counts of the combinations grown in tetracycline agar (no. 3) were essentially the same as the leuconostoc strain

TABLE 3

Counts of streptococci and leuconostocs in tetracycline tomato agar [a]

Streptococci			Leuconostocs		
	Level of tetracycline			Level of tetracycline	
Strain	0	0.15	Strain	0	0.15
		($\mu g/ml$)			($\mu g/ml$)
R1	47×10^7	*	AND	226×10^6	220×10^6
ML-1	25×10^7	*	ZYMR	172×10^6	175×10^6
US-3	30×10^7	*	LYSLE	194×10^6	180×10^6
K-H	62×10^7	*	CR101	141×10^6	133×10^6
C6	74×10^7	*	E-11	129×10^6	123×10^6
E8	105×10^7	*	8356	66×10^7	71×10^7
C13L	180×10^7	*	8359	70×10^7	57×10^7
K	61×10^7	*	CR-6	96×10^6	109×10^6
C10	250×10^7	*	I-11	47×10^7	44×10^7
ML-3	200×10^7	*	9135	54×10^7	49×10^7
924	230×10^7	60×10^3 [c]	8357	49×10^7	42×10^7
C3	62×10^7	*	8358	131×10^7	141×10^7
C13	78×10^6	*	L-169	41×10^7	52×10^7
H1	260×10^6	*	H-3	146×10^6	129×10^6
H4	160×10^6	*	L-2-25	226×10^6	214×10^6
HP	84×10^7	*	H-2-5-10 [b]	31×10^6	39×10^6
926	98×10^7	*	122-5 [b]	85×10^5	74×10^5
Z8L	94×10^7	*			
C1	122×10^7	*			

* No observable growth.

[a] Incubated 48 hr at 30 C

[b] Slow-growing strain; control plate countable 72 hr, tetracycline plate countable 96 hr.

[c] Pinpoint colonies.

TABLE 4
Counts of laboratory-combined starters in tetracycline agar [a]

Strain	Level of tetracycline		Number of isolates	*Leuconostoc*
	0	0.15		
		(μg/ml)		
1 [b] ML-1	250×10^6	0	0	0
2 [c] CR101	141×10^6	133×10^6	3	3
3 M1–1+CR101	244×10^6	150×10^6	27	27
1 [b] R1	47×10^7	0	0	0
2 [c] ZYMR	172×10^6	175×10^6	3	3
3 R1+ZYMR	56×10^7	155×10^6	15	15
1 [b] KH	62×10^7	0	0	0
2 [c] E11	129×10^6	123×10^6	3	3
3 KH+E11	65×10^7	140×10^6	15	15
1 [b] E8	105×10^7	0	0	0
2 [c] 8356	66×10^7	71×10^7	3	3
3 E8 + 8356	112×10^7	56×10^7	29	29
1 [b] C10	250×10^7	0	0	0
2 [c] CR6	96×10^6	109×10^6	3	3
3 C10 + CR6	210×10^7	120×10^6	20	19

[a] Incubated 48 hr at 30 C.
[b] Streptococcus.
[c] Leuconostoc.

alone (no. 2). Numerous colonies were picked from the selective medium and inoculated into tubes of tetracycline tomato broth to insure against possible carry-over contamination of inhibited, but not killed, streptococci. The cultures were incubated 24 hr and identified by microscopic and biochemical examination. Results showed that 105 out of 106 isolations were subsequently identified as *Leuconostoc.*

(d) *Effectiveness of tetracycline on commercial starters.* The effectiveness of the tetracycline agar was then tested with seven mixed starters obtained from commercial laboratories. The cultures were obtained in a lyophilized form and were reconstituted and subcultured two or three times as directed by the supplier to insure maximum activity. Each was then plated in duplicate with the tetracycline and control medium. The plates were incubated at 30 C, one set for 48 hr and the other for 72 hr. Counts are shown in Table 5. The counts on the tetracycline agar increased slightly between 48 and 72 hr, indicating either that some leuconostocs required longer than 48 hr to develop colonies or that the streptococci were not inhibited beyond that period. Colonies from each set of plates were picked and identified as described previously. From the 48-hr plates, 35 out of 35 isolates proved to be *Leuconostoc,* but nine out of 49 from 72-hr plates were identified as *S. lactis.* This indicates that the higher counts with longer incubation were due chiefly to the growth of some streptococci.

(e) *Effect of tetracycline on Streptococcus diacetilactis and enterococci.* Three different strains of *S. diacetilactis* and one strain each of *Streptococcus liquefaciens, Streptococcus faecalis,* and *Streptococcus durans* were tested for growth in the presence of tetracycline. Each strain was plated in tetracycline and in control agar and was incubated at 30 C for 48 hr. In all cases, the control plates had countable colonies within 24 hr, whereas no observable growth occurred in the selective plates before 48 hr.

DISCUSSION

This study has shown that tetracycline inhibition is a simple and practical method for detecting and isolating leuconostocs from mixed lactic cultures. Other antibiotics screened included streptomycin, penicillin, bacitracin, and nisin. Penicillin was also found to be somewhat selective for the leuconostocs, but not to a sufficient degree. The study has indicated possible shortcomings imposed by a failure of the tetracycline agar to inhibit some streptococci longer than 48 hr. The existence of a few slow-growing strains of leuconostocs would appear to make a slightly longer incubation period desirable. However, the authors believe that those slow-growing strains are not likely to have survived continual subculturing and would not normally be found in multiple-strain starters. Additional studies of our entire culture stock revealed that over 90% of the streptococci are incapable of growing to colony size on the selective agar in 48 hr. It was also noted that *S. cremoris* was more inhibited by the tetracycline than *S. lactis.* All *S. cremoris*

TABLE 5

Counts of commercial starters in tetracycline agar

Starter no.	48 hr Level of tetracycline		72 hr Level of tetracycline	
	0	0.15	0	0.15
		(μg/ml)		*(μg/ml)*
1	170×10^6	48×10^5	180×10^6	66×10^5
2	120×10^6	160×10^5	130×10^6	169×10^5
3	46×10^7	13×10^4	47×10^7	18×10^4
4	33×10^7	15×10^4	31×10^7	33×10^4
5	35×10^7	21×10^4	36×10^7	34×10^4
6	58×10^7	*	62×10^7	5×10^3
7	60×10^7	2×10^3	60×10^7	3×10^3

* No observable growth.

strains required three to five days before observable growth occurred.

A 48-hr incubation period should give a highly accurate estimate of the ratio of the leuconostocs to the streptococci. The method should be invaluable for isolating leuconostocs from mixed starters or products, as evidenced by the fact that they were successfully isolated from Cultures 3, 4, 5, and 7 (Table 5), even though the content of leuconostocs was much less than 0.1%. Selectivity, rather than differentiation, enables the method to overcome the problems previously described and should be a useful tool in the study of lactic culture organisms.

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